

AMENDMENTS TO THE SPECIFICATION

IN THE SPECIFICATION

On page 2, line 26, please replace the original paragraph with the following amended paragraph:

-- We have mapped the major integrin recognition sequence of proMMP-9 to be present in the MMP catalytic domain (16). That sequence was mimicked by phage display peptides discovered by biopanning on the integrin α_M I domain, the most active peptide being ADGACILWMDDGWCGAAG (SEQ ID NO: 6) (DDGW) (SEQ ID NO: 1). We have studied here the occurrence of the proMMP-9/ $\alpha_M\beta_2$ complex in PMNs and its role in PMN migration. We found that the complex between proMMP-9 and $\alpha_M\beta_2$ forms already within the gelatinase granules inside the cell and the complex is translocated to the cell surface upon release of the granules during cell activation. Furthermore, a peptide as small as six amino acids in length derived from the MMP-9 catalytic domain was capable of competing with proMMP-9 binding to the β_2 integrin. The hexapeptide and DDGW (SEQ ID NO: 1) both attenuated PMN migration *in vitro* and *in vivo*, suggesting a role for the MMP-integrin complex in PMN motility. --

On page 3, line 7, please replace the original paragraph with the following amended paragraph:

-- Experiments with recombinant MMP-9 domains gave further support for our finding that a site interacting with the integrin is present on the MMP-9 catalytic domain and we developed an active I domain binding peptide that was only six residues in length. This peptide, HFDDDE (SEQ ID NO: 2), corresponds to a linear sequence from the MMP-9 catalytic domain

and efficiently competed with proMMP-9 binding to $\alpha_M\beta_2$ or its purified I domain. The scrambled peptide had no activity, indicating that the order of the negatively charged amino acids is essential for the activity. Similarly to the phage display-derived DDGW peptide (SEQ ID NO: 1), HFDDDE (SEQ ID NO: 2) released cell-bound proMMP-9 and inhibited neutrophil migration *in vitro* and *in vivo*. These results suggest that the proMMP-9/ $\alpha_M\beta_2$ complex is important for neutrophil motility but we cannot exclude the possibility that the peptides also affect other β_2 integrin ligands than proMMP-9. However, the fact that DDGW (SEQ ID NO: 1) and HFDDDE (SEQ ID NO: 2) inhibited the transwell and transendothelial migration of activated neutrophils but not that of resting cells indicates specificity for the action of the peptides. By using CTT, anti-MMP-9 and anti-integrin antibodies, we showed that the peptides inhibited the neutrophil migration that required both proMMP-9 and $\alpha_M\beta_2$. Similarly as with the THP-1 cell line, we thus find that proMMP-9 is a component of the β_2 integrin-directed neutrophil migration at least under these *in vitro* conditions. --

On page 5, line 24, please replace the original paragraph with the following amended paragraph:

-- The cell migration assays revealed two modes of cell motility: β_2 integrin-dependent that was inhibited by DDGW (SEQ ID NO: 1) and other peptides, and β_2 integrin-independent that was not inhibited by the peptides. Thus, it is not surprising that the literature is controversial in terms of the role of proMMP-9 in neutrophil migration. Depending on the experimental models and animal species, some studies have supported protease function in neutrophil migration, whereas others have not. The ability of the cells to show different modes of migration with regard to the stimulus could explain many of the discrepancies. The β_2 integrin- and MMP-independent leukocyte migration may correspond to the observed amoeboid-like movement of leukocytes in 3-dimensional collagen under *in vitro* conditions, which is insensitive to MMP inhibitors. --

On page 6, line 1, please replace the original paragraph with the following amended paragraph:

-- MMP-9 null mice still show neutrophil migration in thioglycolate-induced peritonitis and *in vitro* transmigration of neutrophils across TNF- α -treated endothelial cells. However, MMPs are known to have overlapping functions and other MMPs could compensate for the loss of MMP-9. We have previously found that proMMP-2 complexes with $\alpha_M\beta_2$ and the studies here show that neutrophil MMP-8 can also bind to purified I domain. The HFDDDE (SEQ ID NO: 2) sequence is highly conserved in secreted MMPs and such peptides from many MMPs can bind α_M I domain in a pepspot membrane assay (16, FI 20030923). It remains to be seen which MMP-integrin complexes are functional in the MMP-9 knockout mice. Furthermore, the ability of $\alpha_M\beta_2$ to bind also other proteinases such as elastase and urokinase likely affects neutrophil invasivity.--

On page 6, line 12, please replace the original paragraph with the following amended paragraph:

-- DDGW (SEQ ID NO: 1) and HFDDDE (SEQ ID NO: 2) had potent activities *in vivo* in the mouse peritonitis model, but it is unclear to what extent this was due to inhibition of proMMP-9 as both peptides can potentially inhibit other β_2 integrin ligands as well. A subset of β_2 integrin ligands have a DDGW-like sequence and these include, in addition to MMPs, at least complement iC3b and thrombospondin-1. Our results suggest that the proMMP-9/ $\alpha_M\beta_2$ complex may be part of the neutrophil's machinery for a specific β_2 integrin-directed movement.--

On page 6, line 19, please replace the original paragraph with the following amended paragraph:

-- The present invention is thus directed to new peptide compounds, in specific to a peptide compound comprising the hexapeptide motif HFDDDE (SEQ ID NO: 2). Said compounds can be used as pharmaceuticals, which inhibit neutrophil migration. The inhibitory activity was shown both in *in vitro* and *in vivo* experiments. Consequently, the compounds can be used to prevent and treat inflammatory conditions. --

On page 6, line 25, please replace the original paragraph with the following amended paragraph:

-- The invention thus concerns a compound comprising the hexapeptide motif HFDDDE (SEQ ID NO: 2), and, especially, such a compound for use in inhibiting neutrophil migration, and such a compound for use in prevention and treatment of inflammatory conditions. --

On page 7, line 9, please replace the original paragraph with the following amended paragraph:

-- **Abbreviations:** HMEC, human microvascular endothelial cell; PMN, polymorpho-nuclear neutrophil; CTT, CTTHWGFTLC peptide (SEQ ID NO: 3); CTT W→A, CTTHAGFTLC peptide (SEQ ID NO: 4); LLG-C4, CPCFLLGCC peptide (SEQ ID NO: 5); DDGW (SEQ ID NO: 1), ADGACILWMDDGWCGAAG peptide (SEQ ID NO: 6); HSA, human serum albumin; KKGW (SEQ ID NO: 7), ADGACILWMKKGWCGAAG peptide (SEQ ID NO: 8); LF, lactoferrin; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; GPA, glycolphorin A, TAT-2: tumor-associated trypsinogen-2.--

On page 8, line 17, please replace the original paragraph with the following amended paragraph:

-- **FIGURE 4A to 4D.** α_M -I domain binding to recombinant MMP-9 domains. (Peptides shown correspond to SEQ ID NOS: 2, 9, 1 and 7, respectively)

(4A) Schematic representation of MMP-9 and its recombinant forms produced in *E. coli*. (4B) ProMMP-9, its recombinant forms or BSA were coated on microtiter wells (80 μ g/well) and soluble GST- α_M I domain was allowed to bind at the concentrations indicated. The binding was determined by anti-GST monoclonal antibody. The results are means \pm SD from triplicate wells in this and other figures.

(4C) Binding of proMMP-9 to the immobilized GST- α_M I domain was studied in the presence of each peptide at the concentrations indicated. The binding was determined with the anti-MMP-9 antibody GE-213.

(4D) Binding of GST- α_M I domain to the immobilised proMMP-8, proMMP-9, ICAM-1, and fibrinogen was studied with ICAM-1, DDGW (SEQ ID NO: 1) or KKGW (SEQ ID NO: 7) (50 μ M) as competitors. In control wells, GST was added instead of GST- α_M I domain. The experiment was repeated three times with similar results. --

On page 8, line 31, please replace the original paragraph with the following amended paragraph:

-- **FIGURE 5A to 5D.** Recognition of recombinant MMP-9 domains by $\alpha_M\beta_2$ integrin-expressing cells. (Peptides shown correspond to SEQ ID NOS: 2, 9, 1, 7, 3, and 4, respectively) The studied cells were PMNs (5A, 5B, 5C), $\alpha_M\beta_2$ L-cell transfectants (5D), non-transflectants (5D), and LAD-1 cells (5D). PMNs were in resting state or stimulated with PMA (5A, 5C) or C5a or TNF α (5B) before the binding experiment to proMMP-9 or its domains. Cells were also pretreated with each peptide (50 μ M), antibody (20 μ g/ml) or the α_M I domain as indicated. Unbound cells were removed by washing and the number of adherent cells was quantitated by a phosphatase assay. The experiment was repeated three times with similar results.--

On page 9, line 6, please replace the original paragraph with the following amended paragraph:

-- **FIGURE 6A to 6D.** Blockage of PMN and THP-1 cell migration *in vitro* by gelatinase and β_2 integrin inhibitors. Peptides shown correspond to SEQ ID NOS: 2, 9, 1, 7, 3, and 4, respectively. PMNs (1×10^5 in 100 μ l) were applied on the LLG-C4-GST or GST coated surface (6A) or HMEC monolayer (6B) in the absence or presence of peptides (200 μ M) or antibodies (20 μ g/ml) as indicated. PMNs were stimulated with 20 nM PMA (6A), HMECs with 50 μ M C5a or 10 ng/ml TNF α or left untreated (6B). THP-1 cells (5×10^4 in 100 μ l) were stimulated with 50 nM PMA and applied on the coated surfaces together with each peptide (200 μ M) (6C). The cells migrated through transwell filters were stained and counted microscopically. All experiments were reapeated at least twice. (6D) Phorbol ester-activated THP-1 cells (5×10^4 in 100 μ l) were incubated for 16 h at +37°C in the presence or absence of peptides as indicated. The conditioned medium was analyzed by gelatin zymography. --

On page 9, line 18, please replace the original paragraph with the following amended paragraph:

-- **FIGURE 7A to 7D.** Inhibition of neutrophil migration to an inflammatory tissue. (7A) Mice were injected with thioglycolate or PBS intraperitoneally. The peptides were applied intravenously at the amounts indicated (A). After 3 h, the intraperitoneal leukocytes were harvested and counted. The results show means \pm SD of 2 – 4 mice in a group. (*) indicates statistical significant difference ($p < 0.001$). The experiment was repeated at least 3 times. The infiltrated neutrophils of mice treated with thioglycolate (7B) or PBS (7C) were stained with anti-MMP-9 and anti- α_M , as described in the figure 3 legend. Fluorescence was studied by confocal microscopy. Bars: 9.1 μ m and 4.8 μ m, respectively.

(7D) Gelatinolytic activity of the supernatants from the peritoneal cavities of mice collected as in (7A). Lanes 1-4: samples are from thioglycolate-treated mice; lane 5: a sample from PBS-treated mouse. DDGW (SEQ ID NO: 1), HFDDDE (SEQ ID NO: 2), and DFEDHD (SEQ ID NO: 9) were injected intravenously at doses of 0.1, 0.2 and 0.2 mg per mouse. The arrows show proMMP-9 dimer, proMMP-9 and proMMP-2. The experiment was repeated three times with similar results. --

On page 10, line 27, please replace the original paragraph with the following amended paragraph:

-- The monoclonal antibodies MEM170 and OKM10 are against the integrin α_M subunit (25). The monoclonal anti-MMP-9 antibody (GE-213) was obtained from LabVision (Fremont, CA) and polyclonal MMP-9 from Santa Cruz Biotechnology (Santa Cruz, CA). We also used the previously reported affinity purified antibodies against MMP-9 (3). As monoclonal antibody controls, we used a mouse IgG (Silenius, Hawthorn, Australia) and anti-glycophorin A (GPA) (ATCC). Anti-trypsinogen-2 (TAT-2) antibody was a rabbit polyclonal antibody control (27). The peroxidase-conjugated anti-GST mAb was from Santa Cruz Biotechnology. A rat antibody against the mouse α_M integrin (MCA74) and a FITC-conjugated anti-rat (Fab')₂ were purchased from Serotec (Oxford, UK). The peptides CTT (SEQ ID NO: 3), W→A CTT (SEQ ID NO: 4), LLG-C4 (SEQ ID NO: 5), DDGW (SEQ ID NO: 1), and KKGW (SEQ ID NO: 7) have been described earlier (16, 28). The HFDDDE (SEQ ID NO: 2) and DFEDHD (SEQ ID NO: 9) peptides were custom-made by Neosystem (Strasbourg, France). ProMMP-8 and proMMP-9 were obtained from Calbiochem and Roche, respectively. Diisopropyl fluorophosphate was from Aldrich Chemical Company Inc. (Steinheim, Germany). Human C5a and recombinant TNF- α were purchased from Calbiochem (Biosciences, Inc. La Jolla, CA) and Sigma-Aldrich (St. Louis, MO), respectively.--

On page 17, line 19, please replace the original paragraph with the following amended paragraph:

-- In our previous study, pepspot analysis located the integrin interactive site of proMMP-9 to a 20-amino acid long sequence present in the catalytic domain, QGDAHFDDDELWSLGKGVVV (SEQ ID NO: 10) (see the first priority document). Further screening by the pepspot system has indicated that sufficient integrin binding activity is achieved by truncating this sequence to a hexapeptide, HFDDDE (SEQ ID NO: 2) (data not shown). To confirm that such a short sequence is the bioactive site of proMMP-9, we first prepared bacterially expressed recombinant domains of MMP-9 (Fig. 4A). ΔMMP-9 is composed of the prodomain (Pro) and the catalytic domain but lacks the hemopexin domain. The fibronectin type II repeats (FnII) were also produced as a separate recombinant protein as this is an important substrate-binding region. The procatalytic domain construct ΔMMP-9 bound the α_M I domain nearly as efficiently as the wild type proMMP-9 (Fig. 4B). FnII protein almost lacked activity. The HFDDDE (SEQ ID NO: 2) peptide identified by the solid-phase pepspot analysis was highly active when made by peptide synthesis and inhibited proMMP-9 binding to the α_M I domain with an IC₅₀ of 20 μM (Fig. 4C). The bound proMMP-9 was determined with the GE-213 antibody, which recognizes an epitope of the FnII domain (data not shown). A scrambled peptide DFEDHD (SEQ ID NO: 9) with the same set of negatively charged amino acids was inactive. HFDDDE (SEQ ID NO: 2) was equally potent as DDGW (SEQ ID NO: 1), the α_M I domain-binding peptide discovered by phage display. KKGW (SEQ ID NO: 7), the control peptide for DDGW (SEQ ID NO: 1), was without effect. As the HFDDDE (SEQ ID NO: 2) sequence is highly conserved in the members of the MMP family, we also examined the α_M I domain binding to human neutrophil collagenase, MMP-8. I domain showed a similar DDGW-inhibitable binding to proMMP-8 as to proMMP-9 (Fig. 4D). ICAM-1 and fibrinogen did not compete with either proMMP, implying different binding sites for the matrix proteins and proMMPs in the I domain.--

On page 18, line 10, please replace the original paragraph with the following amended paragraph:

-- After integrin activation, PMNs exhibited an ability to adhere on proMMP-9. PMA-stimulated PMNs bound to microtiter well-coated ΔMMP-9 nearly as strongly as to proMMP-9 (Fig. 5A). Stimulation of PMNs with C5a or TNF- α gave similar results PMN adherence increasing by 3-fold (Fig. 5B). The FnII domain did not support PMN adhesion. PMN adherence was inhibited by HFDDDE (SEQ ID NO: 2) (50 μ M), DDGW (50 μ M), the soluble α_M I domain and the MEM170 antibody (Fig. 5C), indicating β_2 integrin-directed binding. The control peptides (DFEDHD (SEQ ID NO: 9), KKGW (SEQ ID NO: 7)) and an irrelevant monoclonal antibody (anti-GPA) had no effect. The CTT peptide (SEQ ID NO: 3), but not the W \rightarrow A CTT control peptide (SEQ ID NO: 4) lacking MMP inhibitory activity, binds to the MMP-9 catalytic domain (unpublished results) and also inhibited the PMN adherence. MMP-9 antibodies inhibited partially.--

On page 18, line 29, please replace the original paragraph with the following amended paragraph:

-- The *in vitro* migration of PMNs was studied on transwell filter assays. Coating with the artificial β_2 integrin ligand LLG-C4-GST renders cell migration dependent on the β_2 integrins (16, 25). The migration of PMA-activated PMNs was 5-fold in the LLG-C4-GST substratum in comparison to GST substratum (Fig. 6A). HFDDDE (SEQ ID NO: 2) (200 μ M) inhibited the migration of PMA-stimulated cells but not the basal migration of non-activated cells. DDGW (SEQ ID NO: 1), CTT (SEQ ID NO: 3), MEM170 (20 μ g/ml) and polyclonal anti-MMP-9 (20 μ g/ml) worked similarly, affecting the migration of the PMA-activated cells only. Control peptides and an antibody control (anti-TAT-2) had no effect. Similar results were obtained in a

transendothelial migration assay (Fig. 6B). Chemotaxis with C5a or TNF- α increased PMN transmigration by 5-10 fold and inhibition was obtained by DDGW (SEQ ID NO: 1), HFDDDE (SEQ ID NO: 2), and CTT (SEQ ID NO: 3) but not with the control peptides. Similarly, α_M and MMP-9 antibodies inhibited but an antibody control (anti-GPA) did not. We also examined the effects of peptides on THP-1 leukemia cell migration through the LLG-C4-GST coated transwell filters. The results were the same as for PMNs. HFDDDE (SEQ ID NO: 2), DDGW (SEQ ID NO: 1), and CTT (SEQ ID NO: 3) inhibited THP-1 migration and the control peptides did not (Fig. 6C).--

On page 19, line 11, please replace the original paragraph with the following amended paragraph:

-- Previous studies with the DDGW (SEQ ID NO: 1) peptide showed that it can release proMMP-9 from THP-1 cells (FI 2003 0923). We found that the HFDDDE (SEQ ID NO: 2) peptide also released proMMP-9 but was less effective than DDGW (SEQ ID NO: 1) (Fig. 6D). The scrambled peptide did not induce the release of proMMP-9. Under the 16 h incubation time, the peptides had no effect on the secretion of proMMP-2.--

On page 19, line 17, please replace the original paragraph with the following amended paragraph:

-- To study neutrophil migration *in vivo*, we used a mouse model of thioglycolate-induced peritonitis. The cells that infiltrated into the peritoneal cavity within 3 h after thioglycolate irritant were judged to be predominantly PMNs by crystal violet staining. The DDGW (SEQ ID NO: 1) and HFDDDE (SEQ ID NO: 2) peptides had potent *in vivo* activities in this inflammation model (Fig. 7A). An intravenous tail injection of DDGW (SEQ ID NO: 1) or HFDDDE (SEQ ID

NO: 2) inhibited the intraperitoneal accumulation of PMNs. The KKGW (SEQ ID NO: 7) and DFEDHD (SEQ ID NO: 9) peptides used as controls had no effect. The effects of DDGW (SEQ ID NO: 1) and HFDDDE (SEQ ID NO: 2) were concentration-dependent and up to 90 % inhibition was obtained by doses of 50 μ g and 500 μ g per mouse, respectively. DDGW (SEQ ID NO: 1) was active even at 5 μ g given per mouse corresponding to an effective dose of 0.1mg/kg mouse tissue. Approximately 20-fold more PMNs were present intraperitoneally after thioglycolate-stimulus in comparison to the PBS control. The collected inflammatory PMNs stained positively for the proMMP-9/ $\alpha_M\beta_2$ complex by double immunofluorescence (Fig. 7B). The cells collected after PBS injection lacked the complex; they expressed the integrin but had no cell-surface MMP-9 (Fig. 7C). Zymography analysis of the supernatants from the collected intraperitoneal fluid showed that thioglycolate induced elevated levels of gelatinases in comparison to PBS (Fig. 7D). DDGW (SEQ ID NO: 1) and HFDDDE (SEQ ID NO: 2), but not the scrambled peptide, prevented the increase in gelatinase levels in accordance with the inhibition of cell migration. --